Article

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KINOSHITA, Natsuko, *et al*.

Abstract

The stress phytohormone ABA inhibits the developmental transition taking the mature embryo in the dry seed towards a young seedling. ABA also induces the accumulation of the basic leucine zipper (bZIP) transcription factor ABA-insensitive 5 (ABI5) which, apart from blocking endosperm rupture, also protects the embryo by stimulating the expression of late embryogenesis abundant (LEA) genes that conferred osmotolerance during seed maturation. It is unknown whether ABA recruits additional embryonic pathways to control early seedling growth and fitness. Here we identify gia3 (growth insensitive to ABA3), a recessive locus in Arabidopsis mediating cotyledon cellular maturation and ABA-dependent repression of cotyledon expansion and greening. Microarray studies showed that expression of the essential mid-embryogenesis gene Maternal Embryo Effect 26 (MEE26) is induced by ABA during early seedling growth in wild-type (WT) or abi5 plants but not in gia3 mutants. However, we also show that the GIA3 locus controls ABA-dependent gene expression responses that partially overlap with those controlled by ABI5. Thus, the gia3 locus [...]

Reference

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Identification of *growth insensitive to ABA3 (gia3)*, a Recessive Mutation Affecting ABA Signaling for the Control of Early Post-Germination Growth in *Arabidopsis thaliana*

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The stress phytohormone ABA inhibits the developmental transition taking the mature embryo in the dry seed towards a young seedling. ABA also induces the accumulation of the basic leucine zipper (bZIP) transcription factor ABA-insensitive 5 (ABI5) which, apart from blocking endosperm rupture, also protects the embryo by stimulating the expression of late embryogenesis abundant (*LEA*) genes that conferred osmotolerance during seed maturation. It is unknown whether ABA recruits additional embryonic pathways to control early seedling growth and fitness. Here we identify *gia3* (*growth insensitive to ABA3*), a recessive locus in Arabidopsis mediating cotyledon cellular maturation and ABA-dependent repression of cotyledon expansion and greening. Microarray studies showed that expression of the essential mid-embryogenesis gene *Maternal Embryo Effect 26* (*MEE26*) is induced by ABA during early seedling growth in wild-type (WT) or *abi5* plants but not in *gia3* mutants. However, we also show that the *GIA3* locus controls ABA-dependent gene expression responses that partially overlap with those controlled by *ABI5*. Thus, the *gia3* locus identifies an additional arm of ABA signaling, distinct from that controlled by ABI5, which recruits *MEE26* expression and maintains cotyledon embryonic identity. Fine mapping localized the *gia3* locus within a 1 Mb interval of chromosome 3, containing a large DNA insertion of a duplicated region of chromosome 2. It remains unknown at present whether *gia3* phenotypes are the result of single or multiple genetic alterations.

Keywords: Abscisic acid • Arabidopsis • Embryogenesis • Genetics • Germination • Stress response.

Abbreviations: ABI5, ABA insensitive 5, BAC, bacterial artificial chromosome; bZIP, basic leucine zipper; CAPS, cleaved amplified polymorphic sequence; Col, Columbia ecotype; DOG1, DELAY OF GERMINATION 1; GIA3, growth insensitive to ABA; HRGP, hydroxyproline-rich glycoprotein; LEA, late embryogenesis abundant; MEE, maternal embryo effect; TAC, transformation-competent artificial chromosome; SSLP, simple sequence length polymorphism; TAIL-PCR, thermal asymmetric interlaced-PCR; Ws, Wassilewskija ecotype; WT, wild type.

Introduction

The emergence of seeds during evolution facilitated the wide distribution of flowering plants. Quiescent and desiccation-tolerant features of seeds enable plants to pause their lifecycles in the face of unfavorable conditions. The desiccation tolerance of a dry seed is notably implemented by massive accumulation of osmoprotectants during the maturation phase of embryogenesis, which starts after embryo morphogenesis. The accumulation of these proteins, such as late embryogenesis abundant (LEA) proteins, during late embryogenesis is regulated by transcription factors such as ABA-insensitive 3 (ABI3) and ABI5 (Lopez-Molina and Chua 2000, To et al. 2006). At the final stage of embryogenesis, the embryo undergoes programmed desiccation and dormancy, which are positively regulated by the phytohormone ABA and *DELAY OF GERMINATION 1* (*DOG1*), encoding a protein of unknown function (Bentsink et al. 2006).

Upon seed stratification (i.e. chilling and water imbibition in darkness), which breaks seed dormancy, a rapid decrease in the expression of osmoprotectants (e.g. *LEA* gene expression) is observed, which is soon followed by germination (Parcy et al. 1994, Lopez-Molina et al. 2002). The early growth processes—concomitant endosperm rupture and embryonic

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axis elongation (i.e. germination) in turn followed by cotyledon expansion, greening and, finally, onset of vegetative growth—represent a major developmental transition in the life cycle of Arabidopsis.

During the early growth process, the plant faces a fragile transition since it gives up the highly protective state of the mature dry seed in order to develop into a young seedling. Under normal conditions, these steps are completed within 48 h upon seed stratification. However, during this time window, a sudden osmotic stress or exogenous ABA, which signals osmotic stress, severely delays or arrests growth so that the transition to the vegetative seedling state is prevented (Lopez-Molina et al. 2001). Growth-arrested embryos express de novo genes encoding late embryonic proteins such as osmoprotectants (e.g. the *LEA* genes *AtEm1* and *AtEm6*) and retain osmotolerance as long as osmotic stress or ABA is present (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). Thus, when a germinating seedling encounters osmotic stress, its growth will be inhibited and embryonic programs will be reinitiated in response to ABA, thus preventing the plant from precociously entering the vulnerable seedling state.

Importantly, ABA can arrest growth only during a limited time window of about 48 h; once the seedling establishes vegetative growth, ABA is no longer effective to prevent growth. The basic leucine zipper (bZIP) transcription factor ABI5 is required for the stimulation of *AtEm1* and *AtEm6* transcription in response to ABA during the 48 h time window upon stratification, and *abi5* mutants are insensitive to ABA-dependent inhibition of endosperm rupture (i.e. radicle protrusion out of the seed coat) (Finkelstein and Lynch 2000, Lopez-Molina and Chua 2000, Piskurewicz et al. 2008). The molecular genetic processes sustaining this time window of plant developmental plasticity are poorly understood.

To gain more insight about the physiological and developmental role of this time window, we performed a genetic screening to isolate mutants whose germination and early growth is insensitive to ABA. We describe here a novel recessive ABA-insensitive locus: *growth insensitive to ABA3* (*gia3*). The *gia3* locus was fine-mapped to a 1 Mbp region in chromosome 3 where there were no previously reported ABA-insensitive loci. Mapping analysis of the *gia3* locus revealed the existence of a large duplicated fragment of chromosome 2 (5.7 Mbp) inserted in the chromosome 3 interval containing the *gia3* locus.

The study of *gia3* mutants revealed the existence of a novel and specific ABA-dependent signaling pathway that can be phenotypically and molecularly separated from that of *ABI5*. In particular, we found that the expression of the mid-embryogenesis gene *Maternal Embryo Effect 26* (*MEE26*) is induced by ABA in wild-type (WT) and *abi5* plants during the time window, but not in *gia3* mutants. Our results strengthen the notion that ABA-dependent growth arrest is associated with the maintenance of embryonic identity, including cell wall composition and morphology, to protect the plant from environmental stresses. Surprisingly, our studies also revealed that expression of *DOG1*, which positively regulates dormancy (Bentsink et al. 2006), is induced in *gia3* and *abi5* in response to ABA, suggesting the existence of cross-talk between ABA-dependent growth arrest pathways and seed dormancy pathways.

Results

The *gia3* mutation is recessive and behaves as a single genetic locus

Arabidopsis mutants resisting the growth-inhibitory effects of ABA were first isolated by Koornneeff et al. (1984). The original screen focused in identifying *abi* mutant plants whose germination (i.e. rupture of the endosperm) is resistant to high ABA concentrations (Koornneef et al. 1984). Using high ABA concentrations has the drawback of severely delaying germination so that mutations in genes mediating ABA-dependent responses after endosperm rupture (e.g. cotyledon expansion and greening) might be overlooked in the screen.

We wished to identify mutations affecting ABA-dependent responses throughout the entire period of seed germination (i.e. concomitant endosperm rupture and radical protrusion) and post-germination (i.e. further embryonic axis elongation, cotyledon expansion and greening). We lowered the ABA concentrations (3 μM) so that, although delayed, germination eventually takes place, which facilitates monitoring of cotyledon expansion and greening in response to ABA. Mutations obtained in this manner are termed *GIA* (*Growth Insensitive to ABA*). This approach led to the identification of two recessive loci: *gia1*, a novel allele of *abi5* (Lopez-Molina and Chua 2000), and *gia3* (this report).

Phenotypic characterization of *gia3* mutant responses to ABA

Under normal conditions seed germination and early seedling growth proceeded indistinguishably between WT and *gia3* plants (**Fig. 1A**).

Endosperm rupture in *gia3* mutant seeds was moderately, but significantly, insensitive to ABA relative to WT seeds (**Fig. 1B**). For comparison, we also measured endosperm rupture in *abi5* seeds, previously shown to be markedly insensitive to ABA for endosperm rupture (**Fig. 1B**) (Piskurewicz et al. 2008). The double mutant *gia3*/*abi5* was more insensitive to ABA than *gia3* or *abi5* ($P < 0.05$), suggesting that each locus independently participates in repressing endosperm rupture in response to ABA (**Fig. 1B**).

In contrast, *gia3* mutants displayed marked resistance to ABA for cotyledon expansion and chlorophyll accumulation, which was delayed similarly in WT embryos (**Fig. 1A, C**). In the presence of ABA, relative chlorophyll accumulation was comparable in *gia3* and *abi5*, but not in *gia3*/*abi5* double mutants, which accumulated about 2-fold more chlorophyll relative to single mutants (**Fig. 1C**, $P < 0.05$). Thus, each locus independently participates in repressing chlorophyll accumulation in response to ABA (**Fig. 1C**).

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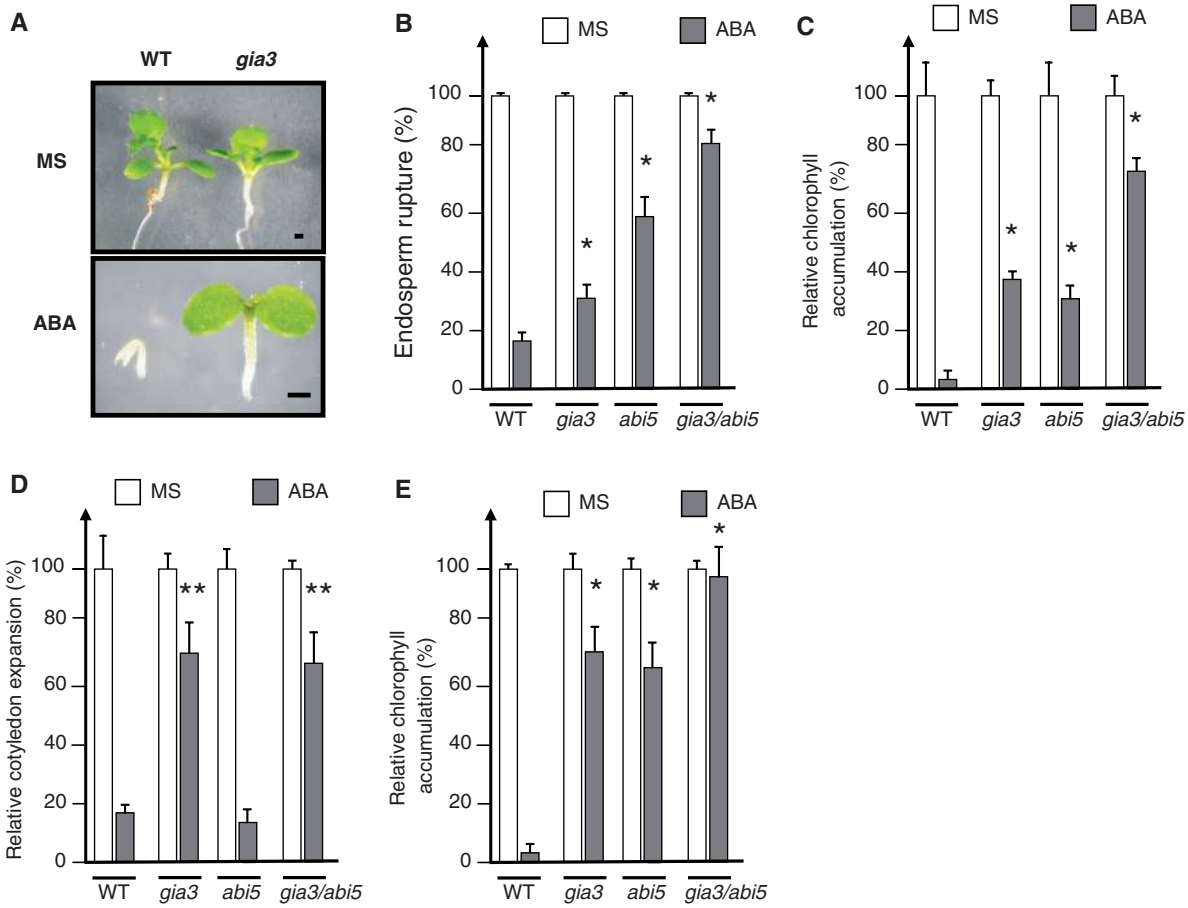

Fig. 1 Isolation and characterization of *growth insensitive to ABA 3* (*gia3*), a novel ABA-insensitive locus. The *GIA3* locus is necessary to repress chlorophyll accumulation and cotyledon expansion in response to ABA. (A) Representative pictures showing WT and *gia3* plants, 7 d after seed stratification in the absence (MS) or presence of 3 μM ABA (ABA). On ABA, the seed coat was removed for better comparison. (B) The percentage of endosperm rupture in populations of WT, *gia3*, *abi5* and *gia3*/*abi5* seeds was scored 72 h after seed stratification in the absence (white bars) or presence of ABA (5 μM, gray bars). Standard deviations are shown ($n \geq 25$). Asterisks indicate a significant difference between the WT and the mutant, based on a two-tailed *t*-test ($P < 0.05$). (C) Relative chlorophyll accumulation in WT, *gia3*, *abi5* and *gia3*/*abi5* seeds 10 d after seed stratification in the absence (white bars) or presence of ABA (5 μM, gray bars). Standard deviations are shown ($n \geq 50$). Asterisks indicate a significant difference between the WT and the mutant, based on a two-tailed *t*-test ($P < 0.05$). (D) Relative cotyledon expansion in WT, *gia3*, *abi5* and *gia3*/*abi5* embryos 48 h after seed stratification and 24 h after transfer to a medium without (white bars) or with ABA (50 μM, gray bars). Standard deviations are shown ($n \geq 14$). Asterisks indicate a significant difference between the WT and the mutant, based on a two-tailed *t*-test ($P < 0.01$). (E) Relative chlorophyll accumulation in WT, *gia3*, *abi5* and *gia3*/*abi5* seeds 5 d after seed stratification and 4 d after transfer to a medium without (white bars) or with ABA (2 μM, gray bars). Standard deviations are shown ($n \geq 25$). Asterisks indicate a significant difference between the WT and the mutant, based on a two-tailed *t*-test ($P < 0.05$).

These observations suggested that *gia3* mutants are specifically deficient in repressing the post-germination steps of cotyledon expansion and greening in response to ABA. This hypothesis was further explored by examining embryo development after transfer to a medium containing ABA upon endosperm rupture (**Fig. 1D, E**). **Fig. 1D** shows that WT cotyledon expansion was strongly delayed by ABA, being about 20% of that observed in the absence of ABA, remarkably, *gia3* mutant cotyledon expansion exhibited pronounced resistance to ABA, being about 70% of that observed in the absence of ABA (**Fig. 1D**). In contrast, *abi5* mutant cotyledon expansion was not resistant to ABA, being comparable with the WT (**Fig. 1D**). Cotyledon expansion in *gia3*/*abi5* double mutant seeds retained the same resistance to ABA as *gia3* single mutant seeds (**Fig. 1D**).

Unlike cotyledon expansion, chlorophyll accumulation was similarly resistant to ABA in both *gia3* and *abi5* mutant seeds (**Fig. 1E**). Moreover, resistance to ABA further increased in *gia3*/*abi5* double mutant seeds, with chlorophyll levels virtually identical to those found in the absence of ABA (**Fig. 1E**, $P < 0.05$).

Taken together, these observations support the notion that ABA-dependent repression of cotyledon expansion is specifically impaired in *gia3* mutants. In addition, ABA-dependent

repression of endosperm rupture and chlorophyll accumulation is separately impaired genetically between *abi5* and *gia3* mutants.

Physical mapping of the gia3 locus

The *gia3* and *abi5* mutants were isolated from a set of 2,400 INRA-Versailles T-DNA lines available from the Nottingham Arabidopsis Stock Center (NASC). The plant transformation vector pGKB5 used in these lines confers resistance to kanamycin in plants.

gia3 [Wassilewskija (Ws) ecotype] was backcrossed into Columbia (Col) and Ws WT backgrounds. The F_2 generation from both crosses established that *gia3* is a monoallelic recessive mutation (data not shown). We found complete co-segregation of kanamycin resistance and *gia3* mutant resistance to ABA in 100 F_2 segregants from the *gia3*×Ws cross (data not shown), indicating that the *gia3* mutation may result from a T-DNA insertion. In parallel, rough mapping with about 100 F_2 segregants from the *gia3*×Col cross located the *gia3* locus to the bottom arm of chromosome 3 between markers SM107-350,0 (16.6 Mb) and ALS (18.0 Mb) (**Fig. 2A**, Materials and Methods). Thus, *gia3* is a previously unreported locus positively mediating ABA responses during germination.

Genomic DNA sequences flanking the T-DNA insertion were isolated by thermal symmetric interlaced (TAIL)-PCR and plasmid rescue techniques (Bouchez et al. 1996, Liu and Whittier 1995). To our surprise, this approach systematically led to two distinct DNA sequences both located in chromosome 2 and separated by 5.7 Mb. We initially interpreted these results as being artifacts from TAIL-PCR and plasmid rescue techniques.

In parallel, fine mapping analysis located *gia3* to an interval of about 1 Mb in chromosome 3 (**Fig. 2A**). However, despite analyzing several thousand segregating plant progeny, no recombination events within the interval could be found, which prevented further fine mapping of the chromosomal location of *gia3* (**Fig. 2A**). We speculated that low recombination might be due to a chromosomal rearrangement present in *gia3* plants, as previously reported in INRA-Versailles lines (Nacry et al. 1998, Laufs et al. 1999, Tax and Vernon 2001, Lafleuriel et al. 2004, Curtis et al. 2009) and often consisting of chromosome fusions joined by T-DNA sequences (Bouchez et al. 1993).

To explore this possibility, we isolated *gia3* DNA genomic fragments containing pGKB5 sequences by screening a *gia3* genomic DNA phage library with pGKB5-derived DNA probes (Materials and Methods). DNA isolated from positive phage clones was subject to restriction map analysis and direct sequencing. Two categories of clones were found, each containing one of the two chromosome 2 sequences discussed above and each fused to chromosome 3 sequences by pGKB5 sequences as shown in **Fig. 2A**. These chromosome 3 sequences

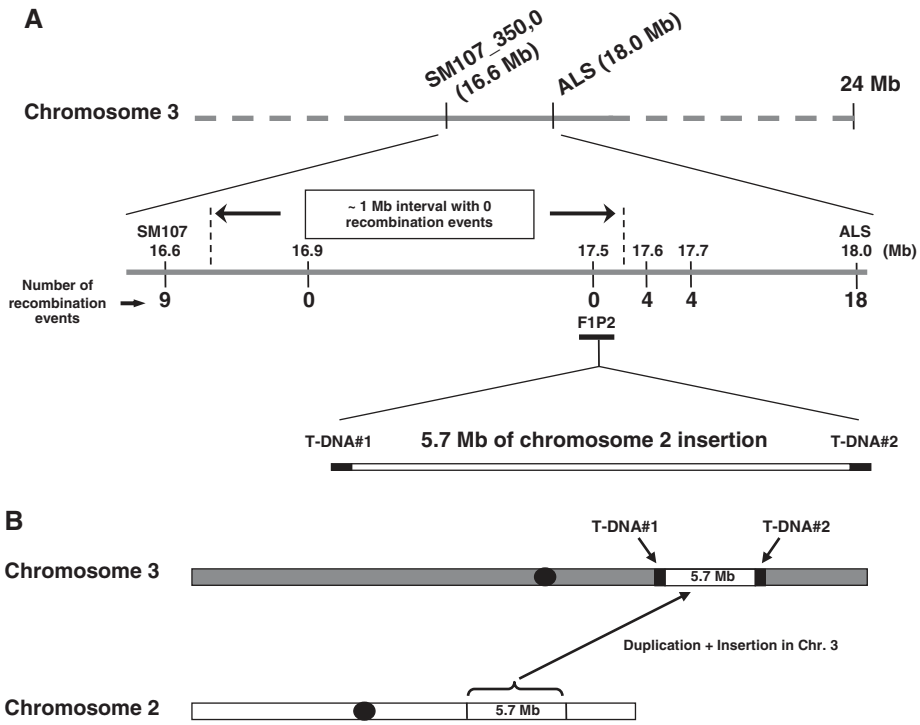

Fig. 2 *gia3* locus characterization. (A) Fine mapping of the *gia3* locus on chromosome 3. Twenty-seven recombinants were isolated between markers SM107_350,0 and ALS. New CAPS and SSLP markers were generated and vertical lines indicate their relative position within the DNA interval containing the *gia3* locus. The number of recombination events is indicated as well as the interval spanned by BAC F1P2 sequences. The site of a 5.7 Mb DNA insertion from chromosome 2 is shown. (B) Model of the chromosome 3 structure of *gia3* showing a 5.7 Mb DNA insertion from chromosome 2 flanked by pGBK5 T-DNA sequences. Black circles indicate the centromere position.

were identical to those of the bacterial artificial chromosome (BAC) F1P2 and belong to the 1 Mb DNA interval fine-mapped as discussed above (**Fig. 2A**). The resulting information combined with Southern blot analyses of *gia3* genomic DNA (**Supplementary Figs. S1, S2**) indicated that a 5.7 Mb duplication of a chromosomal 2 fragment had occurred and was subsequently inserted in the bottom arm of chromosome 3 (i.e. positions 17,565,918 bp and 68,280 bp of chromosome 3 and BAC F1P2, respectively) as depicted in **Fig. 2B**. The chromosome 2 and 3 DNA sequences are linked together by two T-DNA sequences derived from pGKB5 (**Fig. 2B**).

To validate this model further, and to investigate whether other major chromosomal translocations were present in *gia3*, we undertook a chromosome painting approach (Lysak et al. 2001).

Chromosome painting confirms the occurrence of chromosome rearrangements in *gia3*

Five differentially labeled contigs covering chromosomes 2 and 3 were arranged according to the proposed model (**Fig. 3**). Chromosome painting revealed a pattern of staining indeed consistent with the model: a duplication of the region between BAC T30D6 and T28P16 of chromosome 2 translocated between the integration sites of the transgenes within the BAC F1P2 on the bottom arm of chromosome 3 (**Fig. 3**). Painting of all other chromosomes suggested that only chromosome 3 bears major translocation events (**Fig. 3**, **Supplementary Fig. S3**).

The 5.7 Mb chromosome 2 fragment insertion within the *gia3*-containing interval in chromosome 3 could explain the low recombination frequency found in this interval as it may prevent proper chromosomal pairing during meiosis. Moreover, the large insertion may explain why the *gia3* mutant locus is also found in the vicinity of the insertion site. Indeed, previous reports have shown that translocation events can be associated with mutations in flanking sequences in the vicinity of the site of chromosomal translocation (Nacry et al. 1998). Alternatively, the *gia3* mutation may directly result from the chromosome 2 insertion disrupting the expression or coding sequence of one or several genes located in the 1 Mb DNA interval fine-mapped in chromosome 3.

To address these possibilities we analyzed the interrupted sequences of chromosome 3. The 5.7 Mb DNA insertion did not

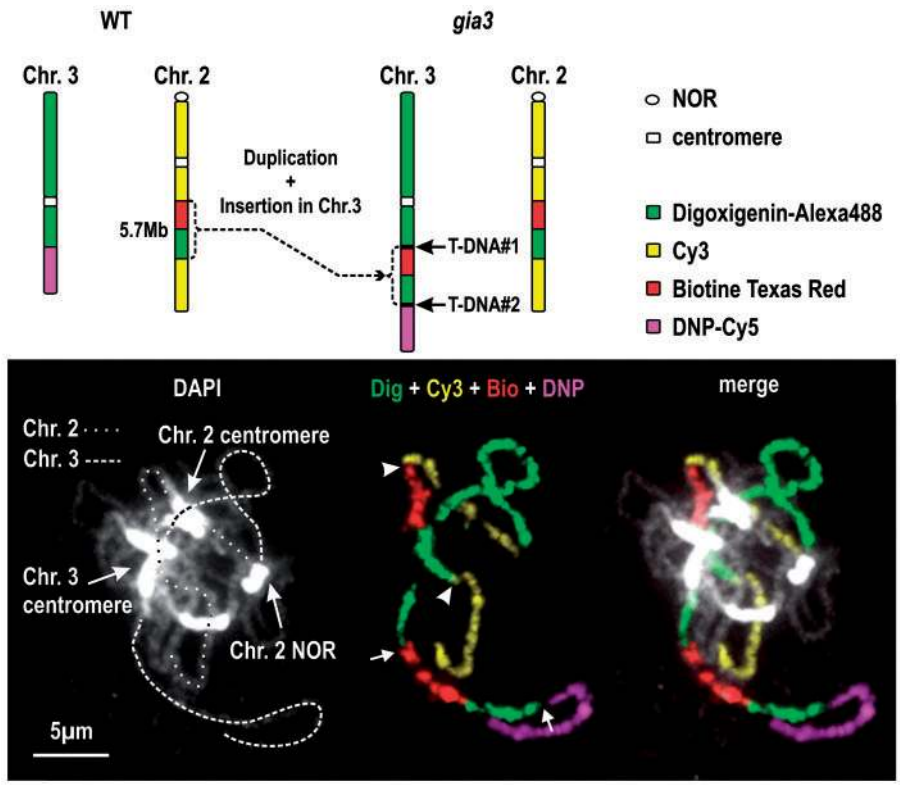

Fig. 3 Visualization of a chromosomal rearrangement event in *gia3* by chromosome painting. Top: schematic representation of the complex probe used to paint chromosome 2 and 3 (Ch. 2 and 3) in the wild type (WT) and in *gia3*. Bottom: chromosome painting with the complex probe on pachytene chromosomes of *gia3*. Left: 4′,6-diamidino-2-phenylindole (DAPI) staining of *gia3* pachytene chromosomes with chromosome 2 indicated with a dotted line and chromosome 3 with a dashed line. Middle: chromosome painting reveals the duplicated region originally from chromosome 2 (region between the arrowheads), translocated with T-DNA on each end, and integrated on the bottom arm of chromosome 3 (region between the arrows). Right: merged picture.

interrupt any predicted gene sequence in chromosome 3. Rather, the fragment is inserted at about 4,000 bp from accessions At3g47610 and At3g47620. Direct DNA sequencing of a 10,000 bp fragment encompassing the insertion site did not reveal DNA sequence alterations in *gia3* (data not shown). Complementation analysis with overlapping genomic DNA fragments covering an interval of about 18,000 bp encompassing the insertion site did not complement *gia3* (data not shown). Taken together, these observations indicate that *gia3* is not located in the near vicinity of the 5.7 Mb DNA fragment insertion site on chromosome 3.

Whole-genome analysis of *GIA3*-dependent gene expression during seed germination

To gain insight into the alterations of ABA-dependent responses in *gia3* mutants during seed germination we compared WT and *gia3* transcriptomes by Affymetrix microarray hybridization (**Supplementary Table S1** and Materials and Methods). To reveal early differences in gene expression between WT and *gia3* plants, seed materials were harvested 48 h after stratification in the presence of ABA, i.e. prior to the onset of obvious developmental differences between WT and *gia3* plants such as cotyledon expansion and greening in *gia3* (**Fig. 4A**, **Supplementary Fig. S4**).

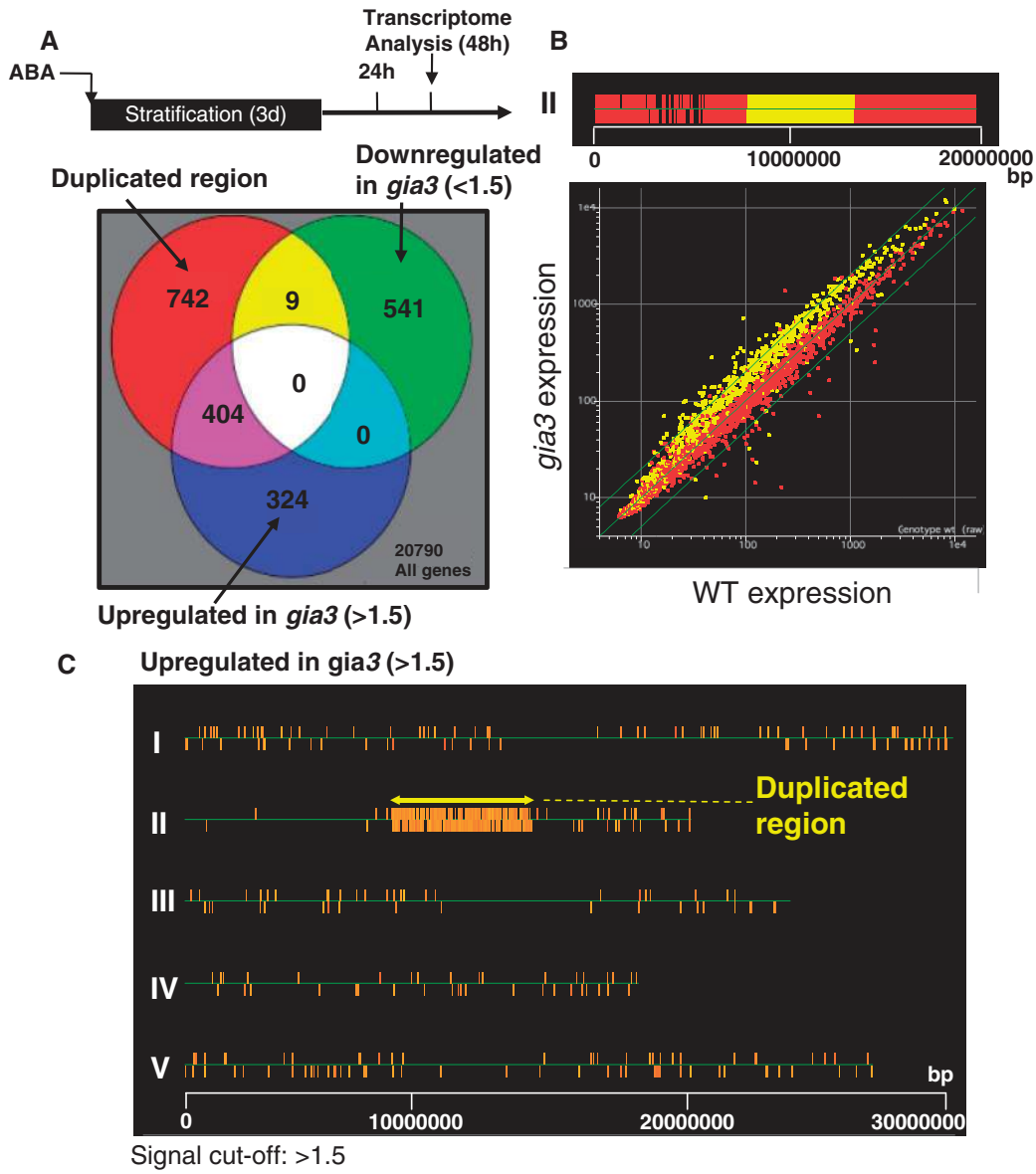

Fig. 4 Whole-genome analysis of *GIA3*-dependent gene expression during seed germination. (A) Venn diagram representation of genes that are present in the duplicated region, up- and down-regulated in *gia3* relative to the WT, respectively (cut-off 1.5-fold). (B) Expression levels of genes in chromosome 2 are compared between *gia3* and the WT. Genes inside the duplicated region are labeled in yellow, and genes outside the duplicated region are labeled in red. (C) Chromosomal representation of the distribution of up-regulated genes (cut-off 1.5-fold). Down-regulated genes are shown in **Supplementary Fig. S5**. Each bar represents a gene; upper and lower bars represent forward or reverse orientation of a gene, respectively, along the chromosome.

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The analysis revealed 550 and 728 transcripts down-regulated (<1.5×) and up-regulated (>1.5×), respectively, in *gia3* (**Fig. 4A**, see **Supplementary Table S1**). As much as 55% of all the up-regulated transcripts belonged to the duplicated region of chromosome 2 (**Fig. 4**). This strong bias most probably reflects the higher gene copy number resulting from the chromosome 2 fragment duplication. The observation also brings further independent support for the model describing the chromosomal translocation present in *gia3* (**Figs. 2, 3**). Moreover, among the down-regulated genes in *gia3*, only nine were located in the duplicated region of chromosome 2, suggesting that no widespread gene silencing in the duplicated chromosome 2 fragment took place in *gia3* plants (**Fig. 4A**, **Supplementary Fig. S5**).

To confirm further the microarray analysis results, we performed Northern blot expression studies using different seed batches and a range of ABA concentrations. For this second round of analysis we chose 30 genes with maximum fold change predicted by microarray. Surprisingly, only those genes identified by the microarray study having a change of >9-fold in expression between WT and *gia3* plants had their expression reproducibly affected in *gia3* in response to ABA (i.e. at least a 2-fold change in Northern blots, see below). This lack of reproducibility could be due to the time chosen to harvest plant material for microarray analysis. Indeed, the WT and *gia3* may be already committed to different developmental paths although they may not yet be visible macroscopically.

Having established that the *abi5* and *gia3* loci control different and overlapping ABA responses during seed germination, we also sought to explore whether *ABI5* also regulates the expression of genes whose expression is altered in *gia3* mutant plants. Among the genes whose expression was initially found by microarray to be affected in *gia3*, we distinguish three categories according to whether their expression is affected: (i) in *gia3* mutants only; (ii) in *abi5* mutants only; or (iii) in both *gia3* and *abi5* mutants.

Table 1 summarizes the results obtained for a final number of 16 genes. Among them, nine were down-regulated and two were up-regulated genes in *gia3* in the presence of ABA with a reproducible fold change of at least 2-fold in Northern blots using different seed batches (see below). Interestingly, among the subset of up-regulated genes located within the 5.7 Mb duplicated region of chromosome 2, the highest fold change predicted by microarray analysis was 6-fold. However, Northern blot analysis revealed a modest, about or less than 2-fold, up-regulation in *gia3* plants (data not shown).

Genes up-regulated in *gia3* mutant seeds

Fig. 5 shows a time course of *DOG1* and ABA-specific *beta-glucosidase1* (*AtBG1*) mRNA expression in WT, *abi5* and *gia3* seeds upon seed stratification in the absence or presence of ABA. *DOG1* promotes seed dormancy and is a member of a small Arabidopsis gene family of unknown function (Bentsink et al. 2006). AtBG1 releases bioactive ABA by hydrolyzing an inactive ABA glucose ester (Lee et al. 2006).

DOG1 was undetectable in WT seeds upon seed stratification under normal conditions, consistent with previous results (Bentsink et al. 2006). Similarly, *DOG1* mRNA was undetectable upon stratification in *gia3* and *abi5* mutants under normal conditions (**Fig. 5**). *DOG1* mRNA expression in response to ABA was not previously characterized. *DOG1* mRNA remained undetectable in WT plants in the presence of ABA; however, *DOG1* mRNA expression was markedly up-regulated in both *gia3* and *abi5*, although to a lesser extent in *abi5* (**Fig. 5**). These results may suggest the existence of a previously unidentified cross-talk between dormancy pathways present in the mature seed and ABA-dependent pathways repressing seed germination and post-germination early growth (see Discussion).

In WT seeds, *AtBG1* mRNA levels were barely detectable upon seed stratification under normal conditions (**Fig. 5**). This is consistent with gene expression databases, which predict

Table 1 Genes differentially expressed in *gia3* and *abi5* mutants

	Fold change (microarray)
Down-regulated in *gia3* only	
MEE26 (At2g34870)	25
Down-regulated in *gia3* and *abi5*	
Epoxide hydrolase, soluble (At2g26750/At2g26740)	57
Cysteine protease inhibitor (At5g05040)	49
F-box protein (acetylglucosamine deacetylase domain) (At1g25210)	42
Meprin and TRAF homology domain-containing protein (At5g26260)	36
Cyclic nucleotide-regulated ion channel (CNGC12) (At2g46450)	21
CBS domain-containing protein (At1g80090)	11
F-box family protein (At1g27580)	10
RNA recognition motif (RRM)-containing protein (At1g73490)	9
Down-regulated in *abi5* only	
ECP-like LEA protein (At3g22490)	6
Oleo4 (At3g27660)	5
Oleo1 (At4g25140)	5
AtEm6 (At2g40170)	4
AtEm1 (At3g51810)	2
Up-regulated in *gia3* only	
β-Glucosidase (AtBG1) (At1g52400)	9
Up-regulated in both *gia3* and *abi5*	
DOG1 (DELAY OF GERMINATION 1) (At5g45830)	20

A list of genes whose expression was up- or down-regulated in *gia3* and further confirmed by RNA blot analysis. Gene expression was also assessed in *abi5* mutants. Fold change indicates the absolute ratio of the hybridization signal between *gia3* and WT plants in the microarray experiment (see **Supplementary Fig. S2**). Gene accession numbers are indicated.

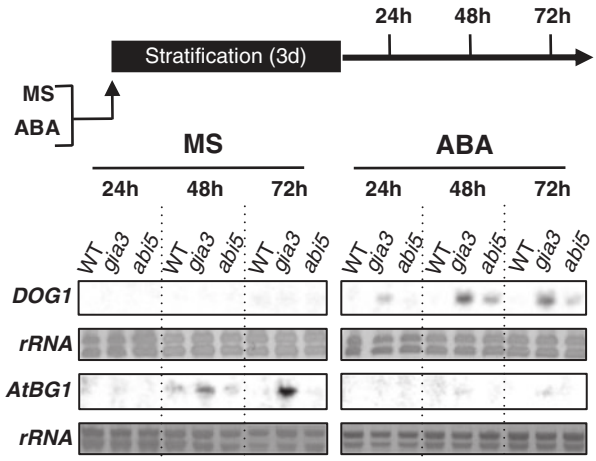

Fig. 5 *DOG1* mRNA expression is up-regulated in *gia3* and *abi5* in response to ABA. *AtBG1* mRNA is specifically up-regulated in *gia3*. RNA gel blot analysis of a time course of *DOG1* and *AtBG1* mRNA levels upon wild-type (Ws), *gia3* and *abi5* seed stratification in the absence (MS) or presence of 5 µM ABA. A 2 µg aliquot of total RNA was used per lane. rRNA, rRNA loading control.

low *AtBG1* mRNA levels upon seed imbibition (Nakabayashi et al. 2005, Schmid et al. 2005, Winter et al. 2007, Goda et al. 2008, Yang et al. 2008). In contrast, *AtBG1* mRNA expression was markedly and specifically up-regulated in *gia3* under normal conditions. In the presence of ABA, *AtBG1* mRNA accumulation was undetectable in WT and *abi5* plants and became markedly lower in *gia3* relative to normal conditions (**Fig. 5**). These data suggest that endogenous *ABA* metabolism during germination is misregulated in *gia3* mutants (see Discussion).

Genes down-regulated in *gia3* mutant seeds

Unexpectedly, among the nine genes whose expression was down-regulated in *gia3* on ABA exposure, eight also had their expression down-regulated in *abi5* (**Table 1**, see examples in **Supplementary Fig. S6** and data not shown). This indicates that common signaling pathways are down-regulated in *gia3* and *abi5* mutant plants during germination.

Only one gene, *MEE26* (At2g34870) encoding a hydroxyproline-rich glycoprotein (HRGP), had its expression specifically down-regulated in *gia3* mutant seeds in the presence of ABA (**Fig. 6A**) (Pagnussat et al. 2005). **Fig. 6A** shows a time course of *MEE26* mRNA levels upon seed stratification in the absence and presence of ABA (5 µM). Under normal conditions, *MEE26* expression was undetectable in dry seeds and thereafter in both WT and *gia3* (**Fig. 6A**). In the presence of ABA, *MEE26* mRNA levels increased between 24 and 48 h, reaching the strongest accumulation at 72 h (**Fig. 6A**). This contrasted with *MEE26* mRNA accumulation in *gia3* mutants, which remained markedly lower at all time points (**Fig. 6A**). The ABA-dependent induction of *MEE26* mRNA accumulation only took place during the same 2 d developmental time window of high responsiveness to ABA previously identified for *ABI5* expression (**Fig. 6B**) (Lopez-Molina et al. 2001).

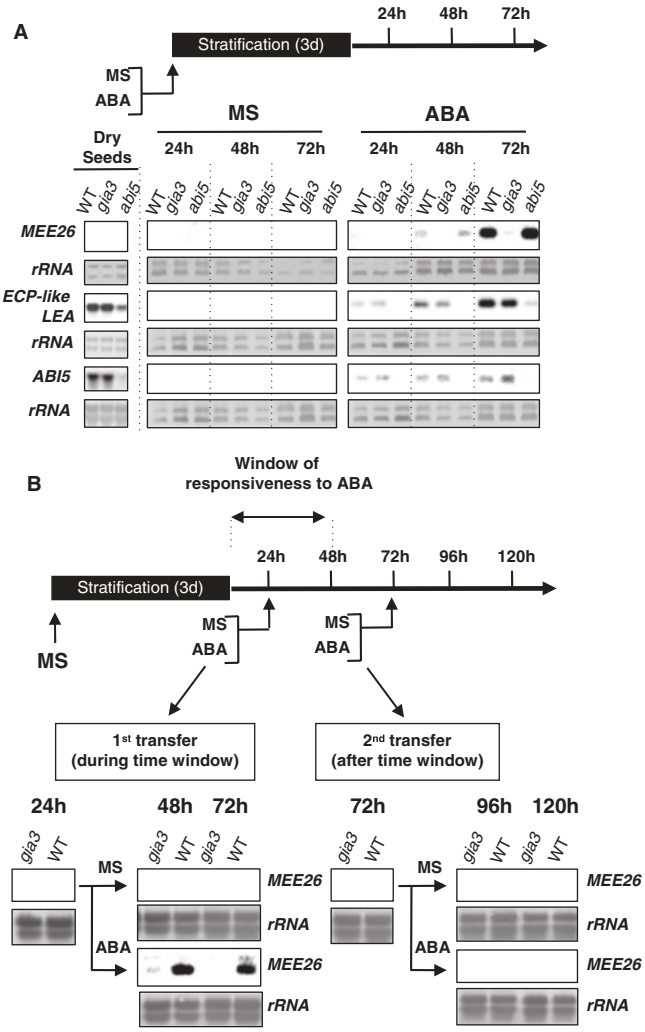

Fig. 6 *MEE26* is specifically down-regulated in *gia3* in response to ABA. *MEE26* expression responds to ABA within the same developmental window previously characterized for *ABI5*. (A) RNA gel blot analysis of a time course of *MEE26*, *ECP-like LEA* and *ABI5* mRNA levels upon wild-type (Ws), *gia3* and *abi5* seed stratification in the absence (MS) or presence of 5 µM ABA. A 2 µg aliquot of total RNA was used per lane. rRNA, rRNA loading control. (B) RNA gel blot analysis of a time course of *MEE26* mRNA upon transfer to medium without (MS) or with 5 µM ABA (ABA) 24 h after stratification (first transfer) or 72 h after stratification (seconds transfer). A 2 µg aliquot of total RNA was used per lane. rRNA, rRNA loading control.

GIA3- and ABA-dependent *MEE26* expression identifies a novel arm of ABA-dependent embryonic gene expression during seed germination

MEE26 was originally identified as a female gametophytic (or maternal) factor which is essential for early embryo development (Pagnussat et al. 2005). Female gametes carrying a *mee26* mutation can be fertilized with WT pollen but the development of the resulting zygote is arrested at the one-cell stage (Pagnussat et al. 2005). *MEE26* expression is restricted to embryogenesis, peaking during mid-embryogenesis according

to database searches (Nakabayashi et al. 2005, Schmid et al. 2005, Winter et al. 2007, Goda et al. 2008, Yang et al. 2008). Our observation that *MEE26* expression is strongly induced by ABA during germination was not previously reported in available transcriptome databases. This could be due to the time points analyzed, which are limited to the first 48 h after seed imbibition (Nakabayashi et al. 2005), and our observation that *MEE26* expression is detected 48 h after seed stratification on ABA (**Fig. 6A**).

Given that *gia3* mutant plants are fertile and viable, we did not anticipate that *MEE26* mRNA expression would be significantly down-regulated during *gia3* mutant embryogenesis. Indeed, *MEE26* mRNA expression was comparable with the WT in *gia3* siliques harvested during early, mid- and late embryogenesis (**Fig. 7A**). This suggested that *MEE26* expression and plant early growth stages are specifically impaired in *gia3* mutants during germination in response to ABA.

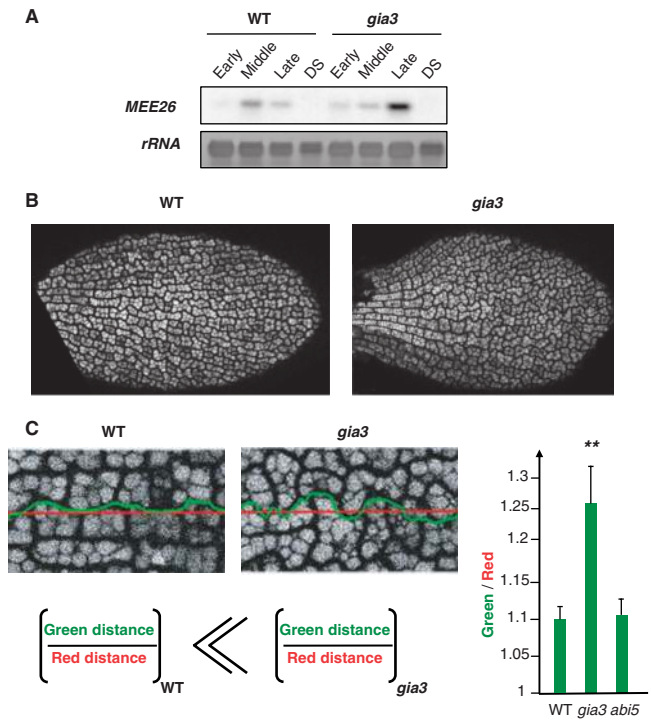

Fig. 7 *MEE26* mRNA expression is not significantly altered in *gia3* during embryogenesis. (A) RNA gel blot analysis of a time course of *MEE26* mRNA in WT and *gia3* siliques at different stages during embryogenesis as indicated. DS, dry seeds. A 10 µg aliquot of total RNA was used per lane. rRNA, rRNA loading control. (B) Representative confocal imaging of cotyledon epidermal cells from WT and *gia3* seeds. Dry seeds were imbibed for 15 min prior to removal of seed coat. (C) Procedure used to quantify the convolutedness of WT, *gia3* and *abi5* epidermal cells. Two point locations in digital confocal pictures are joined by a straight line (red) or by the shortest line (green) that follows the dark, non-autofluorescent path defined by epidermal cell walls. The ratio of the length of green and red lines is used as a measure of cellular convolutedness. Thirty lines per genotype were measured. Standard deviations are indicated. Asterisks indicate a significant difference between WT and *gia3*, based on a two-tailed *t*-test ($P < 0.01$).

We previously reported the occurrence of ABI5-dependent late embryonic gene expression during seed germination in response to ABA, such as that of *AtEm1* and *AtEm6* (Lopez-Molina and Chua 2000, Lopez-Molina et al. 2002). Our microarray analyses initially suggested that *AtEm1* and *AtEm6* are strongly down-regulated in ABA-treated *gia3* seeds (>2- to 6-fold, see **Table 1**, **Supplementary Table S1**) as well as that of *Oleosin1*, *Oleosin4* and *ECP-like*. However, as described above, this could not be confirmed in subsequent experiments and different WT and *gia3* seed batches, which is also consistent with the observation that *ABI5* expression was normal in *gia3* in the absence or presence of ABA (**Fig. 6A**). As expected, *AtEm1* and *AtEm6* mRNA expression was strongly down-regulated in *abi5* mutants in the presence of ABA as well as that of *Oleosin1*, *Oleosin4* and *ECP-like*, which was not previously reported (**Fig. 6A**, **Supplementary Fig. S6C**, **Table S1**). However, *MEE26* mRNA accumulation was strongly stimulated by ABA in *abi5* mutants in a manner indistinguishable from that of WT seeds (**Fig. 6A**).

These data indicate that there is at least an additional pathway of embryogenesis gene expression affected in *gia3* mutants in response to ABA during seed germination. This pathway is separate and distinct from that affected in *abi5* mutants: *MEE26* expression is specifically affected in *gia3* mutants whereas *LEA* expression is specifically affected in *abi5* mutants.

Altered cell wall morphology in *gia3* mature embryos

HRGPs are abundant and key structural components of plant cell walls (Lamport 2001). Therefore, it is conceivable that the *gia3* locus may participate in establishing some features of embryonic cell wall identity in response to ABA during seed germination. The fact that *MEE26* appears to be normally expressed during embryogenesis in *gia3* mutants could reflect the fact that other loci participate in stimulating the expression of *MEE26*, an essential gene, during embryogenesis. However, this does not exclude that the *gia3* locus could exclusively regulate other non-essential aspects of embryonic cell wall morphology during embryogenesis. Thus, the composition, morphology and therefore identity of embryonic cell walls could be altered in *gia3* mutants. To evaluate this possibility, we compared the morphology of the epidermal cells in mature seeds of WT and *gia3* by visualizing their natural cellular autofluorescence using a confocal microscope. **Fig. 7B** shows a representative picture of epidermal cells of cotyledon from mature WT and *gia3* embryos. Cell walls display weak autofluorescence relative to intracellular structures and thus appear as dark lines. Epidermal cells from WT and *abi5* cotyledons were characteristically rectangular in shape and distributed in an orderly way, forming dark lines running parallel to the proximodistal axis of the cotyledon (**Fig. 7B** and data not shown). In contrast, *gia3* cotyledons had fewer rectangular cells, which were mostly located at the base of the cotyledon (**Fig. 7B**).

Moreover, *gia3* epidermal cells progressively assumed, along the proximodistal axis, a distinctive convoluted, puzzle piece-like shape (**Fig. 7B**). This effectively broke the characteristic orderly pattern of parallel lines formed by WT epidermal cells. No significant morphological differences were found between epidermal cells of the WT, *gia3* and *abi5* embryonic axis (data not shown).

To quantify the degree of convolution in WT and *gia3* cells, we took confocal images as shown in **Fig. 7B** and considered two virtual separate points on the surface of the cotyledon and measured the distance separating them in two ways: (i) by measuring the distance of a straight line directly joining them and (ii) by measuring the distance of the shortest path along the dark lines formed by cell wall structures (illustrated in **Fig. 7C**; Materials and Methods). The ratio of these distances provides a measure of cellular convolution. **Fig. 7C** shows that using this procedure *gia3* epidermal cells are significantly more convoluted relative to WT cells, unlike *abi5* epidermal cells. Taken together, these data indicate that the *gia3* locus plays a significant role in seed maturation, at least with regard to mature cotyledon cellular morphology.

Discussion

How ABA is able to repress the numerous and varied developmental processes unfolding during and after germination (i.e. endosperm rupture, radicle elongation, cotyledon expansion and greening) remains incompletely understood. In order to isolate novel loci positively regulating ABA signaling, we followed the classical genetic screen pioneered by Kornneeff et al. (1984). However, we lowered the ABA concentration in the screen to 3 µM ABA while stratifying the seeds for 3 d (Giraudat et al. 1992, Finkelstein 1994, Finkelstein and Lynch 2000, Lopez-Molina and Chua 2000). Under these conditions, WT seed germination is severely inhibited but eventually occurs after a few days (Lopez-Molina et al. 2001). This led to the identification of *abi5* (Lopez-Molina and Chua 2000) and, in the present work, of *gia3* mutants. Comparison of *abi5* and *gia3* seed germination revealed that *gia3* insensitivity to ABA mostly resides at the level of cotyledon expansion (**Fig. 1**), whereas that of *abi5* mostly resided at the level of endosperm rupture (Piskurewicz et al. 2008).

We were unable to identify the nature of the molecular genetic deficiency withstanding the *gia3* recessive locus using standard map-based approaches. This is due to the extremely low recombination frequencies taking place in the chromosome 3 interval where *gia3* is located. This is most probably due to the large chromosome 2 fragment insertion in that interval, which is best visualized in a chromosomal painting experiment (**Fig. 3**). We attempted to identify *gia3* by examining the phenotypes of mutant SALK lines harboring T-DNA insertions within the *gia3* mapping interval (see **Supplementary Table S2**). This approach did not give tangible results and was not pursued further due to the large number of genes within the interval and the fact that we were uncertain whether the *gia3* phenotype would be easy to follow in a different ecotype.

Given the size of the genomic DNA interval in which *gia3* is located, we attempted a transformation-competent artificial chromosome (TAC)-based complementation approach (data not shown). TAC binary vectors allow the transfer of genomic DNA fragments as large as 100 kbp. We had very limited success with this technique, succeeding with a single successful TAC insertion (TAC K6M3) containing a 68,000 bp fragment of chrosomome 3 spanning the insertion site of the 5.7 Mbp DNA insertion of chromosome 2 (see **Supplementary Fig. S7A**). *gia3*/*K6M3* transformants were found to have normal ABA sensitivity during germination as well as normal *MEE26* expression in response to ABA and normal cell wall morphology (**Supplementary Fig. S7B, C**). These data therefore suggest that the *gia3* locus is located within a 68,000 bp DNA interval spanning position 20,156 to 87,890 or chromosome 3 as indicated in **Supplementary Fig. S7A**. However, given that only one *gia3*/*K6M3* transformant was obtained, we consider this result to be preliminary. We also attempted to identify a DNA fragment within the TAC K6M3 clone that could complement *gia3* in transformation experiments. **Supplementary Fig. S7A** summarizes the collection of fragments characterized. Taken together, they cover 61% of the 68,000 bp genomic fragment of chromosome 3 in TAC K6M3. None of these fragments complemented *gia3*. Clearly a combined approach of complementation analysis (using smaller genomic fragments) and direct sequencing of *gia3* genomic DNA should lead to the identification of a putative single *GIA3* gene. However, we cannot exclude that the large chromosome 2 fragment inserted in chromosome 3 affects multiple DNA locations in complex manners. This could include alterations in the expression of multiple genes necessary to implement ABA-dependent responses during germination.

As a result of these difficulties, the salient findings of this work are based on the phenotypic characterization of *gia3* mutants during seed germination. They collectively indicate the existence of an ABA-dependent pathway controlling embryogenesis gene expression during germination and embryo maturation that is distinct from that controlled by ABI5. Indeed, we showed that (i) ABA-dependent repression of cotyledon expansion and greening does not take place in the single recessive locus *gia3* and (ii) that the same mutant locus prevents the ABA-dependent and high mRNA expression of *MEE26*, a gene encoding a putative HRGP cell wall protein previously shown to be essential for embryo survival during mid-embryogenesis (Pagnussat et al. 2005). In contrast, *gia3* mutants accumulate normal *MEE26* mRNA levels during embryogenesis (**Fig. 7**), which most probably accounts for their survival.

Thus, a notable consequence of our studies on *gia3* is to reveal that the positive regulation of *MEE26* expression by ABA is developmentally restricted to the previously identified time window of high ABA responsiveness occurring during seed germination (Lopez-Molina et al. 2001).

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MEE26 encodes a putative HRGP factor and at this stage of the investigation we can only speculate about the developmental and physiological significance of inducing its expression during germination in response to ABA. HRGPs are structural components of cell walls (Lamport 2001) and this led us to speculate that the *gia3* locus may regulate some other aspects of cell wall morphology during embryogenesis although not necessarily linked to *MEE26*, since it is normally expressed in *gia3* during embryogenesis. Indeed, consistent with this hypothesis, we found that the regulation of epidermal cellular organization and morphology in the mature cotyledon is specifically altered in *gia3* mutants (**Fig. 7**). In this respect, it is also notable that the main resistance of *gia3* to ABA concerns cotyledon expansion.

These observations may suggest that in *gia3* mutants, the normal establishment of the embryonic character of cotyledon epidermal cells is altered. In turn, this may explain the failure of the *gia3* mutant to repress cotyledon expansion in response to ABA, if one assumes that this is a process efficiently implemented when epidermal cell walls maintain an embryonic molecular composition.

As stated above, *abi5* and *gia3* mutants were isolated in the same genetic screen (Lopez-Molina and Chua 2000). *ABI5* encodes a bZIP transcription factor positively regulating the expression of osmotolerance *LEA* genes in the dry seed and during the time window of ABA responsiveness taking place during seed germination. Moreover, in the case of *abi5* mutants, resistance to ABA occurs at the level of endosperm rupture. Thus, the characterization of *gia3* extends previous observations with *abi5* mutants showing that ABA plays a role in promoting (i) normal seed maturation; (ii) germination and early growth arrest; and (iii) temporally restricted embryonic gene expression. Thus, the study of *gia3* mutants identifies an arm of embryogenesis gene expression that is distinct and separate from that involving *LEA* gene expression, which is positively regulated by ABA and ABI5 (see model in **Fig. 8**). It is therefore tempting to speculate that the *gia3* mutant locus affects the activity or expression of a transcription factor. These data bring further support to the notion that a time window of about 2 d allows the plant to acquire an embryonic protective character and arrest growth in response to environmental stresses (Lopez-Molina et al. 2001).

Our work may reveal unexpected insights into the regulation of seed dormancy pathways. *DOG1*, encoding a protein of unknown function, is a positive regulator of seed dormancy (Bentsink et al. 2006). In WT seeds, *DOG1* mRNA is detected in dry seeds and then decays upon imbibition under normal conditions (Bentsink et al. 2006). *DOG1* mRNA levels are higher in *abi5* and *gia3* mutant mature seeds but also decay upon seed imbibition under normal conditions (**Fig. 5, Supplementary Fig. S6B**; Nakabayashi et al. 2005, Schmid et al. 2005, Winter et al. 2007, Goda et al. 2008, Yang et al. 2008). Here we showed that ABA does not significantly affect *DOG1* mRNA expression upon imbibition of WT seeds, which was not previously investigated (**Fig. 5**). We also observed that *DOG1* mRNA expression is strongly induced by ABA in *abi5* and *gia3* mutants. Since the dry seed is also a state associated with higher ABA levels, these data could collectively suggest that *ABI5* and the *GIA3* locus participate in repressing *DOG1* expression in response to ABA.

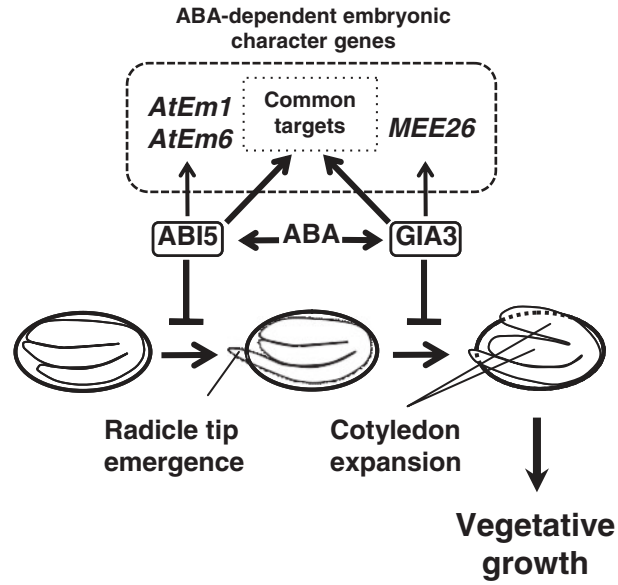

Fig. 8 A model for ABI5- and GIA3-dependent germination and early growth arrest.

The mechanisms of seed dormancy remain poorly understood; however, it is widely accepted that ABA is essential to maintain the seed in a dormant state (Finch-Savage and Leubner-Metzger 2006). It is therefore tempting to speculate that at least a subset of genetic loci positively regulating ABA responses are also implicated in an ABA-dependent negative feedback loop operating during establishment of seed dormancy to limit the extent of *DOG1*-dependent seed dormancy (Bentsink et al. 2006).

Moreover, our findings regarding *AtBG1* expression illustrate the complexity of the regulation of ABA metabolism during seed germination in response to environmental cues. It was recently proposed that stress increases endogenous ABA levels by recruiting the activity of AtBG1 to deconjugate stored pools of ABA glucose ester (Lee et al. 2006). Our observation that *AtBG1* expression is repressed in response to ABA in WT seeds could also suggest a negative feedback mechanism that limits endogenous ABA levels. Moreover, the fact that *AtBG1* expression is specifically high in *gia3* in both the absence and presence of ABA indicates that a single genetic locus controls the negative feedback limiting endogenous ABA levels in germinating seeds. This possibility is rather intriguing since it would imply that the same locus exerts two functions which are apparently contradictory: on the one hand it is necessary to arrest growth in response to ABA and on the other it also limits endogenous ABA accumulation.

Materials and Methods

Plant material

Arabidopsis thaliana ecotype Ws and Col were used as WT plants. *gia3* and *abi5-4* were isolated in the T-DNA INRA-Versailles lines (stock Nos. N5389 and N5455) from the NASC.

Germination assays

Plants were grown, harvested and stored according to Piskurewicz et al. (2008). The germination assay were done according to Perruc et al. (2007). For cotyledon area measurements, seedlings were dissected under a stereo microscope using a blade on syringes, and dissected cotyledons were placed flat on nylon mesh. Pictures were taken using a digital camera attached to a stereo microscope and the cotyledon area was measured using ImageJ software (NIH).

Plasmid constructs and plant transformation

DNA manipulations were performed according to standard methods (Sambrook et al. 1989).

Mapping of the GIA3 locus

The *gia3* mutant was outcrossed to Col WT plants and mapped as described (Lopez-Molina and Chua 2000). For fine mapping, novel single SSLP (simple sequence length polymorphism) and CAPS (cleaved amplified polymorphic sequence) markers were generated and are available upon request.

Chromosome painting

Pachytene chromosomes were isolated from fixed immature flower buds of *A. thaliana* ecotype Ws and *gia3* according to Lysak et al. (2001). Contiguous BACs obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH, USA) and selected for negligible amounts of repeats (Lysak et al. 2003) were pooled for painting individual *A. thaliana* chromosome regions. BAC DNA isolation, labeling by rolling circle amplification or nick translation, and fluorescence in situ hybridization (FISH) were performed as described (Berr and Schubert 2006, Berr et al. 2006).

DNA and RNA isolation and analysis

DNA and RNA isolation was performed as previously described (Vicient and Delseny 1999, Perruc et al. 2007). Southern blots of genomic DNA and RNA were performed according to standard procedures (Sambrook and Russell 2001).

Genomic library construction

The *gia3* genomic library was constructed using a Lambda FIX II/XhoI Partial Fill-In Ventor Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Probes for Southern blot were generated by PCR amplification using O65, gaactgcaggacgaggcagcg; and O66, gataccgtaaagcacgaggaagc.

Microarray analysis

For probe labeling and hybridization, total RNA extracted from *gia3* and WT seedlings was treated with DNase RQ1 (Promega, Madison, WI, USA) and cleaned up using Qia pure columns (Qiagen, Valencia, CA, USA). Total RNA from each pool was used to synthesize biotinylated cRNA according to the Affymetrix protocol (kits from Invitrogen for cDNA synthesis and Enzo for biotinylated cRNA synthesis). A 20 µg aliquot of biotinylated cRNA was hybridized to a mouse Affymetrix Arabidopsis Genome ATH1 array.

Supplementary data

Supplementary data are available at PCP online.

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